# DECLARATION (A)

SIR:

I, Shunichi KURODA declare that:

1) I am one of the inventors of the above-identified application, and am familiar with the subject matter of said application as well as the disclosures in the cited references.

2) In order to demonstrate the advantage of the present invention, the following experiments were carried out under my direction and supervision.

## Experiment 1

The purpose of this Experiment was to determine whether drugs, in particular, low-molecular weight compounds (in this case, Calcein) is encapsulated in or outside of the HBsAg L protein particle (hereinafter referred to as "BNC-L (Bio-Nano-Capsule L protein particle)" prepared in the same manner as Example A of the specification.

BNC-L (200 µg/ml) and Calcein (0.25mM, 0.5mM, 1mM) in PBS were allowed to stand at room temperature for 10 minutes for dissolution. The solution (100 µl) in cuvette was electroporated by applying pulses twice of either 50V, 750 µF or 150V, 750 µF.

The Calcein/BNC-L solution obtained after electroporation was treated with 1 mM $CoCl_2$ (Group B), treated with 10mM DTT and 2.5% SDS (Group C) or not treated (Group A). RFU (485/520) of the resulting solutions was calculated by the following method:

(Calculation Methods)

- RFU(485/520) Measurements of Calcein/BNC Alone ・・・ Group A (Un-processed Group)

- RFU(485/520) Measurement after Addition of 1mM $CoCl_2$ solution ・・・ Group B (Quenching outside calcein)

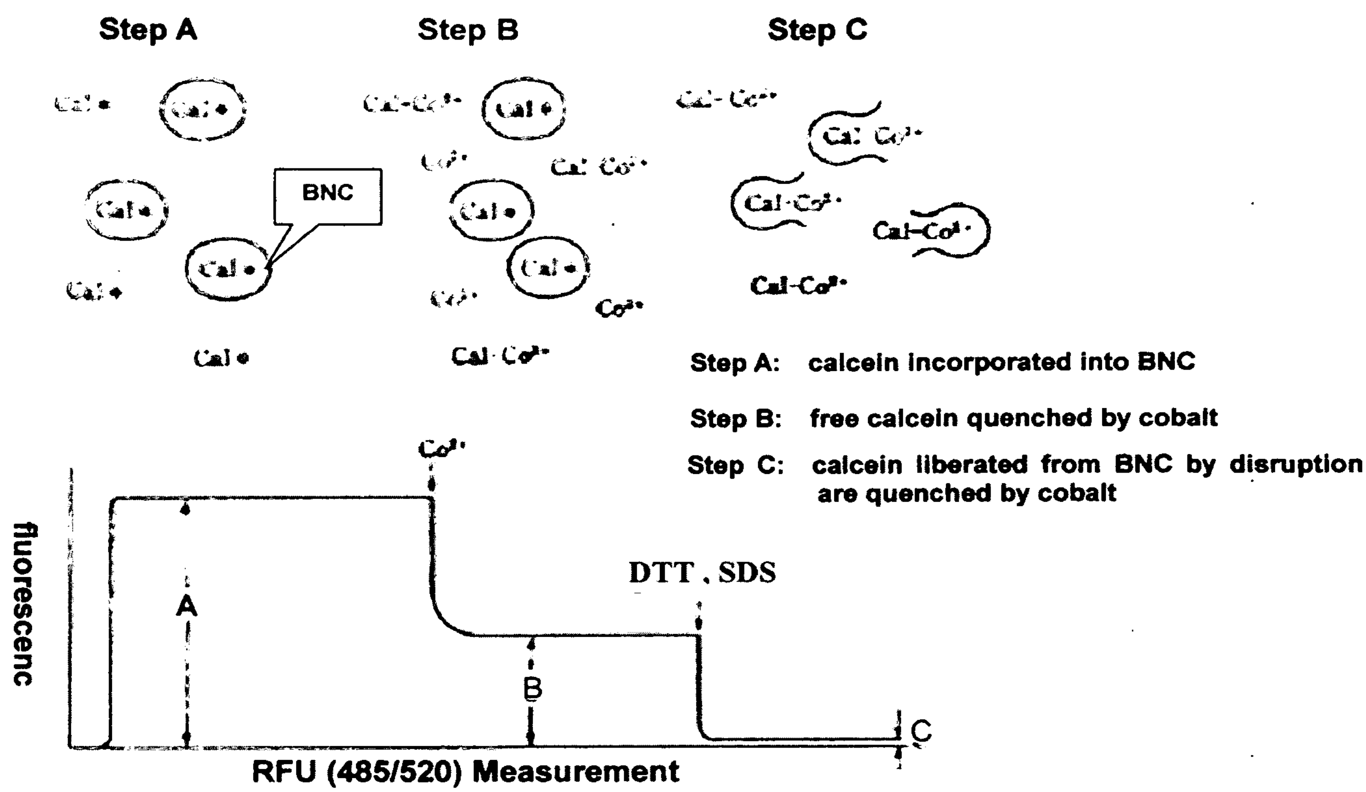
- RFU(485/520) Measurement after addition of 10mM DTT and 2.5% SDS ・・・ Group C (Releasing calcein from the particles, then quenching with cobalt)

(Calculation theory)

The rate of encapsulation can be calculated using Cobalt' s quenching properties for calcein.

・ Cobalt ions quench fluorescence of calcein at low concentration

・ In theory when one cobalt ion bonds to one molecule of calcein, fluorescence is quenched completely, therefore enabling us to measure the fluorescence of calcein within BNC particles.




A = RFU(485/520) Measurements of Calcein/BNC Alone

B = RFU(485/520) Measurement after Addition of 1mM $CoCl_2$ solution

C = RFU(485/520) Measurement after addition of 10mM DTT and 2.5% SDS

$$\text{Rate of Calcein Encapsulation} = \frac{(B - C)}{(A - C)}$$

The Results and Calculations of the Number of Encapsulated Calcein Molecules in BNC Particles are shown below.

I, the undersigned, declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: Aug 19 '09

Shunichi KURODA

## Results

| | Dilution Rate | Group A Calcein RFU(485/520) | | Group B Add. Of CoCl2 RFU(485/520) | | Group C Add. Of DTT, SDS RFU(485/520) | | Calculated Enclosed Amount | | Average | Calcein Conc. (mg/ml) | Average (mg/ml) | Average (ug/ml) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Calcein/BNC Without EP RT | 1 | 49792 | 49641 | 9958 | — | 2288 | 1772 | 7668 | — | 7668 | 0.01392 | 0.0009 | 0.8531 |
| | 2 | 47575 | 47635 | 1212 | 612 | 333 | 332 | * | 280 | 280 | 0.001005 | | |
| | 4 | 42934 | 43329 | 267 | 310 | 153 | 161 | 114 | 149 | 131 | 0.000832 | | |
| | 8 | 36775 | 35455 | 161 | 149 | 112 | 106 | 49 | 43 | 46 | 0.000622 | | |
| Calcein/BNC 50V RT | 1 | 48321 | 48211 | 8690 | 7531 | 1528 | 1687 | 7162 | 5844 | 6503 | 0.011804 | 0.002 | 2.0396 |
| | 2 | 46881 | 46841 | 1558 | 1282 | 482 | 490 | 1076 | 772 | 924 | 0.003344 | | |
| | 4 | 43307 | 42556 | 513 | 527 | 279 | 270 | 234 | 257 | 245 | 0.00176 | | |
| | 8 | 36041 | 35113 | 282 | 269 | 204 | 201 | 78 | 68 | 73 | 0.001014 | | |
| Calcein/BNC 150V RT | 1 | 47652 | 47692 | 7552 | 11069 | 2187 | 2561 | 5365 | 8508 | 6936 | 0.012591 | 0.003 | 3.2539 |
| | 2 | 48409 | 46425 | 2246 | 1740 | 742 | 682 | 1504 | 1058 | 1281 | 0.004641 | | |
| | 4 | 43067 | 42701 | 835 | 876 | 443 | 441 | 392 | 435 | 413 | 0.002981 | | |
| | 8 | 35972 | 35025 | 532 | 494 | 367 | 358 | 165 | 136 | 150 | 0.00214 | | |

yellow lines: data omitted due to insufficient cobalt quenching. *: data omitted due to experimental error. —: no data, mean ± SD

## Calculations

| | Calcein Concentration(mg/ml) (ug/ml) | Calcein Concentration Molar Conc. (μM) | Protein Concentration WT (ug/ml) | Protein Concentration Molar Conc. (μM) | BNC Particles Molar Conc. (μM) | No. of Calcein Molecules per one BNC particle |
|---|---|---|---|---|---|---|
| Calcein/BNC Without EP | 0.8531 | 1.3703317 | 297.68 | 5.724615385 | 0.052041958 | 26.3 |
| Calcein/BNC 50V | 2.0396 | 3.276202715 | 280.261 | 5.389634615 | 0.048996678 | 66.9 |
| Calcein/BNC 150V | 3.2539 | 5.226728777 | 277.418 | 5.334961538 | 0.04849965 | 107.8 |

**From the data and calculations shown on the previous page, it is deduced that inside each bio-nanocapsule there are following numbers of encapsulated calcein molecules:**

| Conditions | No. of Calcein Molecules |
|---|---|
| **Without Electroporation** | About 30 |
| **50V, 750uF, Two Pulses** | About 70 |
| **150V, 750uF, Two Pulses** | About 100 |

**Calcein molecules are encapsulated in BNC particles.**

## DECLARATION (B)

SIR:

I, Shunichi KURODA declare that:

1) I am one of the inventors of the above-identified application, and am familiar with the subject matter of said application as well as the disclosures in the cited references.

2) In order to demonstrate the advantage of the present invention, the following experiments were carried out under my direction and supervision.

<u>Experiment (DXR Direct Encapsulation)</u>

The purpose of this Experiment was to determine whether drugs, (in this case, Doxorubicin Hydrochloride) can be directly encapsulated into the HBsAg L protein particle (hereinafter referred to as a "BNC-L (Bio-Nano-Capsule L protein particle") prepared in the same manner as in Example A of the specification.

<u>Used method:</u>

pH Gradient Method (Simple diffusion)

A method using the shift in molecule ↔ ion dissociation equilibrium of the drug to be encapsulated, by pH change

<u>Used drug:</u>

DXR: Doxorubicin Hydrochloride (M.W. = 543)

An anti-malignant tumor agent (anticancer agent)

<u>Experiment Procedures:</u>

1. BNC-L lyophilized from a citrate buffer (pH 4.0) was reconstituted with ultrapure water (1 mg/ml). The internal and external solution had a pH of 4.0.

2. Gel filtration using PBS(-) (pH 7.2). By gel filtration, external buffer was exchanged for PBS buffer (pH 7.2), thereby establishing a pH gradient across the membrane between the interior and exterior of the particles.

3. Addition of the substance to be encapsulated; i.e., doxorubicin or

a cyclodextrin/doxorubicin composite at the concentration indicated below.

Doxorubicin (DXR): 1 mg/ml.

Gamma-Cyclodextrin/doxorubicin composite (CyD-DXR):

CyD at 2.6mg/ml and DXR at 1mg/ml

(Cyclodextrin has an ability to incorporate doxorubicin into its inner space.)

4. These mixtures were incubated at 60°C for 30 min. Then, free-DXR was removed by gel filtration.

5. An aliquot of DXR-encapsulated BNC-L was applied on a gel filtration column (a PD-10 column). Then, 0.5 ml of the buffer was sequentially added to the column, and each fraction was recovered. The encapsulated DXR concentration was measured for these fractions and samples before gel filtration. The difference in fluorescence intensities (RFU (485/584)) before and after the addition of DTT + Triton X (for the disruption of particles) was determined as the DXR concentration using a microplate reader.

Results:

1. Incorporation of free DXR into HBsAg L protein particle

The results for free doxorubicin are shown in the following Fig. 1 and Table 1.

Fig. 1

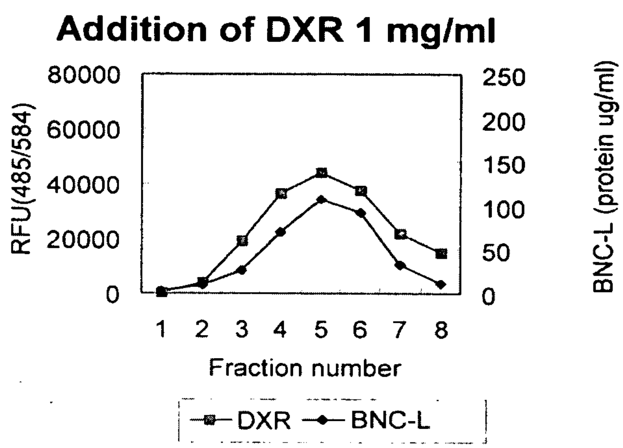



Table 1

| | 1 mg/ml | Encapsulated DXR (mg/ml) | Encapsulation ratio (%) |
|---|---|---|---|
| BNC 1mg | DXR | 0.059 | 5.9 |

The amount of DXR encapsulated in 1 mg/ml of BNC

The elution of DXR alone was slow in the gel-filtration column, and the free DXR was seen in fraction #15 and later (data not shown). On the other hand, using the BNC treated with DXR, the elution profile of the protein concentration of BNC-L by gel-filtration coincides well with that of the DXR concentration (Fig. 1); that is, the higher the protein concentration, the higher the DXR concentration. The results indicated that DXR is incorporated into BNC-L. The concentration of DXR before the fractionation was 0.059 mg/ml (encapsulation rate 5.9%) in this particular experiment (Table 1).

<u>2. Incorporation of cyclodextrin/DXR composite into HBsAg L protein particle</u>

Fig. 2

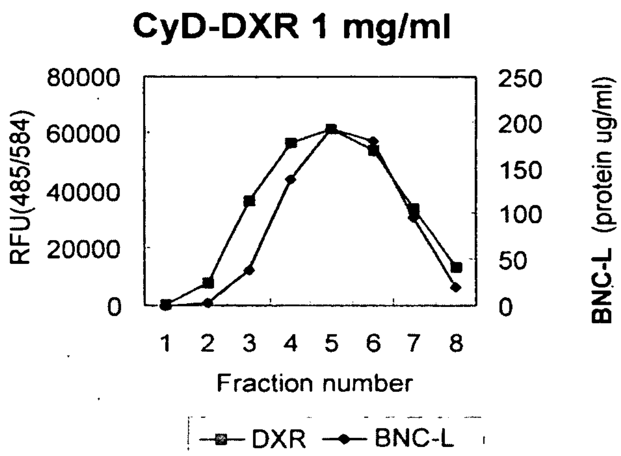



Table 2

| | 1 mg/ml | Encapsulated DXR (mg/ml) | Encapsulation ratio (%) |
|---|---|---|---|
| BNC 1mg | DXR-CyD | 0.106±0.0133 | 10.6 |

n=3 (mean±SD). The amount of DXR encapsulated in 1 mg/ml of BNC

The encapsulation rate of free DXR was low, presumably due to the leakage of encapsulated DXR. It is reported that a CyD/DXR composite can be encapsulated into a liposome in a degree comparably similar to that of free DXR, while the leakage of DXR in composite form is low (Ref. 1).

To elevate the amount of DXR encapsulated into BNC-L, we next tested the encapsulation of the CyD/DXR composite. As shown in Fig. 2, the elution profile of the protein concentration of BNC-L by gel-filtration coincides well with that of the DXR concentration; that is, the higher the protein concentration, the higher the DXR concentration. The results indicated that DXR in composite form is incorporated into BNC-L. The concentration of DXR before the fractionation was 0.1058 mg/ml as an

average of three experiments, and the encapsulation rate was 10.6% (Table 3)

Conclusion:

The results show that small molecules such as doxorubicin and a CyD/DXR composite can be encapsulated into HBsAg L protein particles by pH gradient (i.e. a form of simple diffusion). It is likely that molecules of a molecular weight of at least 1,800 can be encapsulated into HBsAg L protein particles, since the components of the composite have a molecular weight of 1,297 for gamma-CyD, and 543 for DXR.

References

1. Prolonged Retention of Doxorubicin in Tumor Cells by Encapsulation of γ-Cyclodextrin Complex in Pegylated Liposomes. Y. Hagiwara, H. Arima, F. Hirayama, K. Uekama, J. Incl. Phenom. Macrocycl. Chem., 56, 65-68 (2006).

I, the undersigned, declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: Aug 19 '09

Shunichi Kuroda

Shunichi KURODA

# DECLARATION (C)

## Delivery of micro beads and plasmid DNA using BNC-ZZ

Many diseases, such as angina, rheumatoid arthritis, and infections are related with inflammation. Selectins are proteins expressed on the surface of vascular endothelial cells, and play roles as cell adhesion molecules in inflammatory state. To deliver substances to vascular endothelial cells, anti-selectin antibodies were attached to BNC-ZZ (bio-nanocapsule with ZZ-tag), and the delivery of beads-loaded BNC-ZZ or plasmid DNA-loaded BNC-ZZ were examined in two inflammatory disease models.

### Experiment 1

Delivery of beads by BNC-ZZ attached anti-P-selectin antibody in collagen-induced arthritis mice.

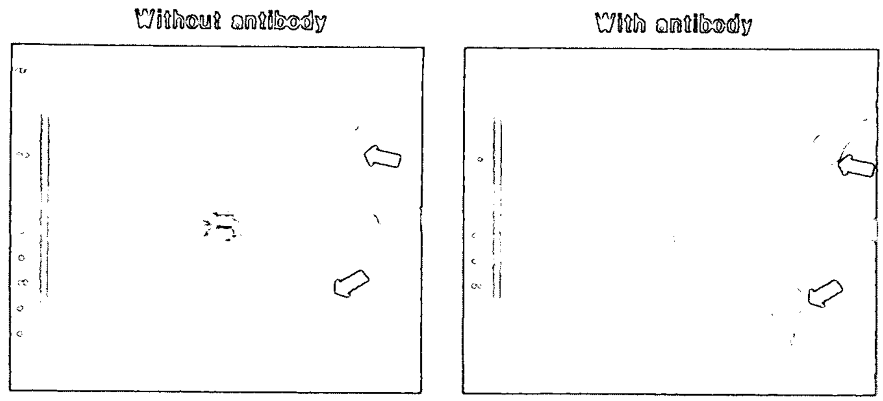
【Methods】

Fluorescent beads (Fluosphere 100nm, Molecular Probes) solution (0.2%, 2ml) was added to a vial containing freeze-dried liposome (61 mg/vial, Coatsome EL-01-A , Nichiyu) to from a bead-liposome conjugate. The conjugate of 6 μL was diluted with 94 μL of distilled water, and added to a vial containing freeze-dried BNC-ZZ of 100 μg. These procedures produced a complex of BNC-liposome containing fluorescent beads. This complexes of 100 μg (as BNC protein) was incubated with 20 μg of anti-P-selectin antibody (BD Pharmingen) for 15 min, and then with a coupling reagent, BS3 (Pierce) for additional 40 to 60 min. The final product is the anti-P-selectin antibody-displaying BNC-liposome complex containing fluorescent beads.

Mice were administered with a mixture of Type-II collagen and equal amount of H37Ra adjuvant to their foot pad twice with 3 weeks interval to make a collagen-induced arthritis model.

【Result】

The model mice were injected with anti-P-selectin antibody-displaying BNC-liposome complex containing fluorescent beads from tail vein at 10 μg/mouse (as BNC protein), and the fluorescence of lower half of the animal was observed using an in vivo imaging system (OV100, Olympus). As a control, some animals were injected with no antibody-displaying BNC-liposome complex containing fluorescent beads. As seen in Fig 1, the injection of P-selectin displaying complex showed much higher fluorescence in the foot pads where the main inflammatory reaction occurs as observed at 24 hrs after injection.




(10μg/mouse injection after 24hrs)

Fig.1 Accumulation of fluorescence in inflammatory food-pad after the i.v. injection of complexes of BNC(ZZ)-liposome containing fluorescent beads with or without anti-P-selectin antibody.

## Experiment 2

Delivery of plasmid DNA into target cells by E-selectin-displaying BNC-ZZ in experimental autoimmune uveoretinitis (EAU) mice.

〔Methods〕

DNA solution (250 μg/ml, EGFP vector, CLONTECH) of 1 ml was added to a vial containing freeze-dried liposome (1.51 mg/vial, Coatsome EL-01-D, Nichiyu) to from a DNA-liposome conjugate. The conjugate of 66.7 μL was diluted with 33.3 μL of distilled water, and added to a vial containing freeze-dried BNC-ZZ of 100 μg. These procedures produced a complex of BNC-liposome containing DNA. This complex of 100 μg (as BNC protein) was incubated with 1 μg of anti-E-selectin antibody (BD Pharmingen) for 15 min, and then with a coupling reagent, BS3 (Pierce) for additional 40 to 60 min. The final product is the anti-E-selectin antibody-displaying BNC-liposome complex containing DNA. The procedure to produce beads-loaded BNC was described in experiment 1.

Mice were administered s.c. with emulsion mixture of M. tuberculosis (6.0 mg/ml) and Freund's complete adjuvant containing IRBP peptide (GPTHLFQPSLVLDMAKVLLD) at 200 μg (as the peptide weight). They received an additional sensitization by injecting i.p. an emulsion of Freund's complete adjuvant containing 0.1 μg of pertussis toxin. Mice developed grade II EAU (Thurau et al. 1997, Clin Exp Immunol) was employed for further experiments as examined by slit-lamp examination.

〔Result〕

The model mice expressed high level of E-selectin antigen in the blood vessels of whole area of

the retina as examined by immuno-histochemistry using the anti-E-selectin antibody (Fig.2). The observations with con-focal laser microscopy using retinal whole mount system (Chang-Ling 1997, Microsc. Res. Tec.) revealed that the i.v. administration of anti-E-selectin displaying BNC-ZZ labeled with Cy7 fluorescent dye, or conjugated with fluorescent beads-containing liposome resulted in higher fluorescence at endothelial cell regions of retinal vessels in EAU mice than those with no-antibody displaying BNC-ZZ.
Next, the anti-E-selectin antibody-displaying BNC-liposome complex containing DNA was injected i.v. to the EAU mice at 10 μg/mouce (as BNC protein). As a control no antibody-displaying BNC-liposome complex containing DNA was used. Three days after injection, the retinal whole mounts were prepared and observed with the con-focal microscopy. In the group injected with the anti-P-selectin antibody-displaying complex showed green fluorescence derived from GFP in the endothelial cell regions of retinal vessels(Fig 3), while in the control group, no fluorescence was observed.

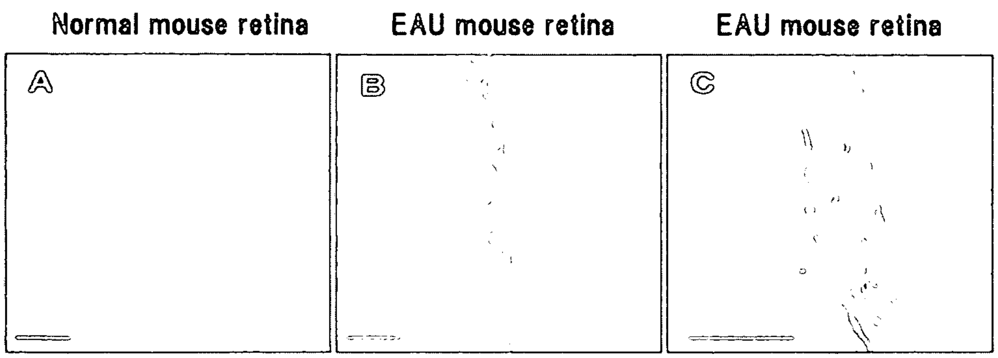



Fig.2 Presence of E-selectin on the retinal vascular endothelial region in EAU mice.

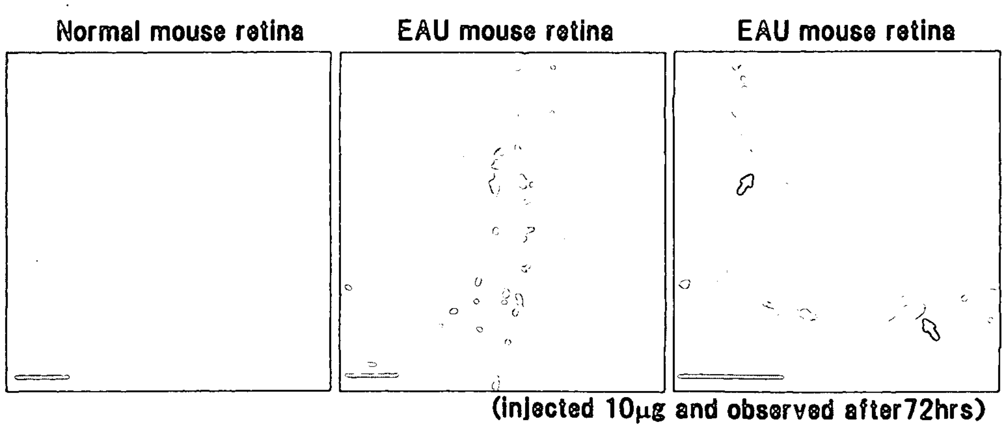



Fig.3 Delivery of GFP in the retinal vascular endothelial region in EAU mice after i.v. injection of BNC-liposome complex.
Arrows indicate GFP-expression cells showing no signal in nucleus area.

【Conclusions】

These results indicate in the inflammation models, anti-P- and E-selectin antibodies are effective to induce antibody-dependent cell recognition and delivery of substances when used with BNC-ZZ.

Date: Aug 19 '09

Shunichi KURODA